

The use of supramolecular structures as protein ligands

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Abstract Congo red dye as well as other eagerly self-assembling organic molecules which form rod-like or ribbon-like supramolecular structures in water solutions, appears to represent a new class of protein ligands with possible wide-ranging medical applications. Such molecules associate with proteins as integral clusters and preferentially penetrate into areas of low molecular stability. Abnormal, partly unfolded proteins are the main binding target for such ligands, while well packed molecules are generally inaccessible. Of particular interest is the observation that local susceptibility for binding supramolecular ligands may be promoted in some proteins as a consequence of function-derived structural changes, and that such complexation may alter the activity profile of target proteins. Examples are presented in this paper.

Keywords Congo red · Supramolecular ligand

Introduction

The search for new drugs and protein reagents usually involves single organic molecules which attach to protein

binding sites. Such studies are widely supported by computer-aided drug design tools [1, 2], however ongoing developments in supramolecular chemistry have directed the attention of researchers toward new types of reagents, formed by assembling several distinct chemical compounds. These reagents are potentially attractive due to their unique properties and possible applications in various fields [3–13]. From the medical and biological point of view interaction of supramolecular structures with proteins is a topic of great interest, yet — unfortunately — our knowledge of this phenomenon remains limited. Interaction of this type is known to occur in cell membranes; however surface interaction with proteins (such as in the cell membrane) is not sufficient to facilitate biological function. Penetration of a ligand composed of assembled molecules into the protein interior seems necessary. Out of many possible supramolecular architectures rod-like or ribbon-like organization appears to be the most promising for this purpose due to the existence of partly exposed hydrophobic portions of assembled compounds, favoring adhesion. Congo red is perhaps the best recognized self-assembling dye of this type and hence commonly used as a model [14–17]. It is a known amyloid stain but it also appears to form complexes with structurally unstable proteins, such as abnormal IgG light chains derived from serum or urine of myeloma patients [18–23]. Molar excess of this dye usually attaches to proteins and while a large fraction of the bound dye may easily be removed by adsorption, some dye usually remains, suggesting penetration and anchorage within the protein body. A question therefore arises: should penetrating molecules be treated as assemblages of individual units or as an integral ligand? Support for the latter interpretation is provided by studies on some foreign compounds, e.g., rhodamine B intercalating into supramolecular Congo red and penetrating, together with this dye, into a protein for which it has no affinity by itself [24]. Moreover, the observed correlation of

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self-assembling tendencies of different organic dyes and their capability for protein complexation strongly favors treating supramolecular ligands as coherent units [25, 26]. An integrated supramolecular ligand seems necessary to achieve penetration into the protein interior. This paper discusses some effects and implications of supramolecular Congo red complexation with proteins.

Congo red as a model supramolecular dye

Interaction of proteins with ligands is usually limited to a binding site at a specific location in the protein molecule. Binding sites are commonly found as cavities in the protein body facilitating contact of the ligand with the hydrophobic interior and separating the ligand from direct dissociation pressure of the surrounding water solution. The specificity and strength of interaction which allows ligand-protein complexation is due to the specific shape of the binding site accommodating the ligand as well as to proper distribution of binding groups.

The strength of non-specific low-contact interaction of organic compounds outside the binding site is generally insufficient to stabilize ligation, even if penetration into the protein interior is occasionally possible. There is, however, an exception to this rule. It concerns self-assembled molecules creating rode-like or ribbon-like supramolecular structures. They are usually formed by elongated, planar aromatic ring-containing organic molecules with (possibly) symmetric distribution of charges in the molecule. Congo red and related dyes are an example (Fig. 1) [27–33]. Theoretical calculations enable modeling of such supramolecular structures, based initially on semi-empirical techniques and then using *ab initio* parameterization [17, 27]. In contrast to the molecular organization of supramolecular micellar structures with a standard globular shape, where the hydrophobic portions of amphipatic molecules are basically hidden in the micelle, fibrillar structures allow significant exposure, promoting adhesion. This property is further

enhanced by structural plasticity due to non-covalent stabilization. Pieces of such ribbon-like supramolecular structures consisting of several or more assembled molecules have been confirmed to penetrate into proteins and bind as single ligands. β -plates in proteins appear to be the most favored acceptor [26, 34–36]. Dye ligands penetrate preferentially in between the polypeptide strands of β -plates. Replacement of the least stable strand (away from the packing locus) to make room for the dye ligand is a frequently observed phenomenon (Fig. 2) [37, 38]. Well-packed proteins are mostly impervious to dye penetration and — generally speaking — certain structural instabilities seem essential to render the protein capable of binding supramolecular ligands. Instability may occasionally be natural, as in the case of the so-called disordered proteins [39], but more often arises as a result of protein misfolding [40–42], mutation-derived structural alteration [22] or the presence of unfolding conditions [28, 38].

Function-derived secondary structural changes

In some cases proteins become susceptible to penetration by supramolecular ligands as an effect of structural changes generated in the protein molecule by its function-related activities. This implies that primary structural changes in the protein molecule caused by the ligand itself are often connected with some distant secondary alterations and packing instability which allows Congo red penetration and binding [16, 36, 43–45]. In these cases complexation of supramolecular ligands arrests the primary structural changes connected with the protein's function. The mechanism of this process corresponds to a known type of enzymatic inhibition called uncompetitive inhibition [46]. In the case of enzymes, strong binding of substrates without simultaneous release of products interferes with biological function, causing inhibition. Function-related binding of dye may be studied, e.g., on the basis of known complexes involving acute phase serum proteins. Figure 3 shows the distribution of serum proteins separated by two-dimensional agarose electrophoresis with Congo red added to the second (perpendicular) run (placing the dye below the starting line of proteins) to select and expose proteins interacting with the dye [47]. The rapidly migrating dye accelerates migration of proteins involved in its binding. As a result such proteins outpace the diagonal distribution of proteins which do not engage in binding dye. Selective binding of dye to serum proteins associated with various diseases indicates that proteins exposed by the dye (i.e., those located above the diagonal) may have been altered by pathological processes. This is likely due to complexation of abundant disease-related products. Location of these particular proteins in electrophoretic fractions hints at the nature of the

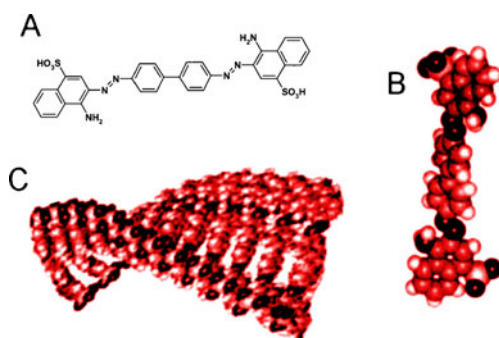
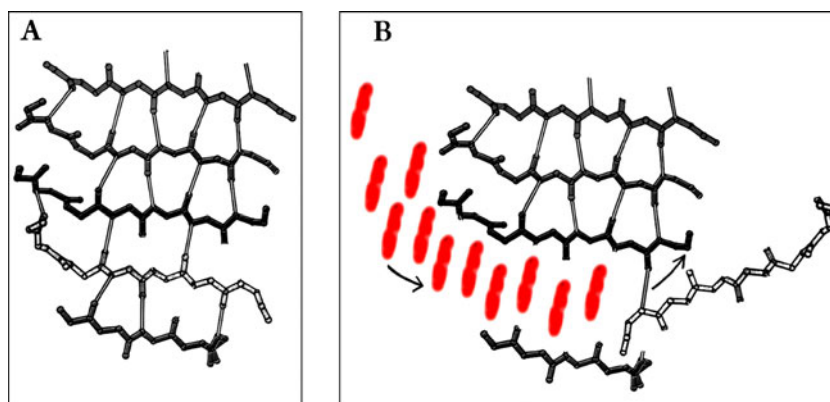


Fig. 1 Congo red—single molecules (a, b) and supramolecular form of the dye (c) [17, 27]

Fig. 2 Schematic view of supramolecular Congo red penetration into a locus of decreased stability in a β -plate. Replacement of one β -strand by the dye (**a**, **b**)



abnormal state in the organism. The phenomenon of function-derived generation of protein susceptibility for Congo red binding is of great interest for theoretical and practical use in biology and medicine.

Immune complexation as the mechanism generating structural alterations allowing Congo red binding

Antibodies and immune complexation fall squarely within the focus of our interest. Complexation of antibodies with large insoluble antigens (cells, bacteria) generates strains and structural changes in molecules, rendering them susceptible to Congo red binding.

The strains arise as a consequence of fitting and complexation of noncomplementary objects represented here by bivalent antibodies on the one hand, and by randomly distributed antigenic determinants on the other (Fig. 4) [48]. This effect concerns only complete bivalent antibodies and neither Fab nor even (Fab)₂ complexation is capable of producing it [43, 44]. Nevertheless, secondary structural alterations in the antibody molecule (caused by antigen ligation) increase its affinity for Congo red and, as a result, promote formation of immune complexes [43, 44, 49–51]. This enhancement effect caused by Congo red binding to immune complexes formed by complete, bivalent antibodies was measured in the SRBC-antiSRBC system using polyclonal antibodies isolated by Sephadex gel filtration and

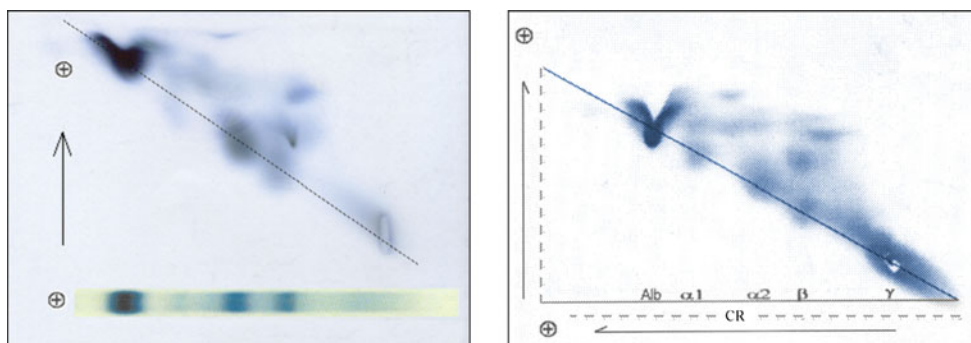
labeled by iodine-125. The curve clearly indicates the increased number of antibody molecules bound to red cell following increases in Congo red concentration (Fig. 5). Experiments reveal binding of low-affinity antibodies, naturally present in polyclonal sera, removable without Congo red by washing.

The site of Congo red binding is localized in immunoglobulin V domains but outside of the antigen binding site [16, 37, 38]. Constraints destabilize the packing of the N-terminal polypeptide chain fragment in the V domain. Destabilization further propagates to elbow regions, enabling rotational movements of polypeptides in the CH1 domain and, as can be expected, favoring complement fixation. These movements are interpreted as intramolecular signaling [16, 47, 52]. Structural instabilities in V domain favor Congo red penetration. The dye replaces the unstable terminal polypeptide fragment in the packing locus. Theoretical modeling, confirmed by experimental observations, reveals structural changes associated with the binding of supramolecular ligands. [44, 51, 52] A schematic representation of this phenomenon in the V domain of L chain is presented in Fig. 6.

The use of Congo red as a target-directed carrier

The Congo red ligand, composed of several dye molecules derived from a ribbon-like micelle, penetrates into the

Fig. 3 The effect of using two-dimensional agarose electrophoresis of serum proteins with Congo red dye fast migrating added to the second; perpendicular electrophoretic run in order to distinguish (by way of faster migration) proteins which become susceptible to binding the dye due to disease-modified function-related structural changes



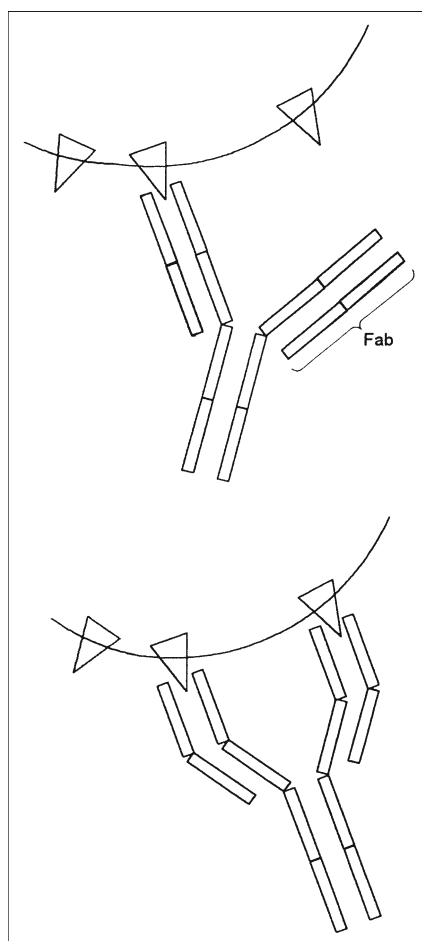


Fig. 4 Schematic presentation of the mechanism explaining generation of constraints in bivalent antibody molecules upon binding to antigen

protein body. While its binding strength is rather high, a portion continues to protrude from the protein molecule, creating an external anchor point to which more dye can attach itself. As a result, the protein becomes a carrier for a large quantity of dye. The excess of dye associated with the protein-dye complex may be removed by gel filtration (using bio-gel) or brief reduction by sodium dithionate. Confirmation for these predictions was provided by EM analysis of the isolated by SephadexG200 gel filtration fraction involving IgG molecules aggregated by heating in the presence of Congo red. The picture shows individual or clustered IgG molecules as white irregular spots, loaded with an excess of Congo red, preventing the precipitation of partly unfolded (by heating) protein molecules (Fig. 7) [28, 53]. The non-covalently stabilized supramolecular material attached to the protein may additionally bind foreign compounds, including drugs, by intercalation. In this way foreign compounds with no affinity for the given protein may be introduced to it as “payload” carried by supramolecular dye. Immune complexes which selectively bind Congo red may therefore represent an attractive target model for compounds potentially carried by supramolecular ligands. Congo red with rhodamine B bound by intercalation was used as the medium for SRBC-antiSRBC agglutinating system to verify the validity of the above mechanism. Agglutinates were studied in UV using a fluorescence microscope [54]. Antibodies were not added to the control sample. Samples were washed carefully to remove the medium containing free dye molecules. Microscope imaging fully confirms that immune complexes act as a receptor system which binds Congo red and therefore becomes the carrier for the included rhodamine B (Fig. 8).

Fig. 5 Increasing the affinity of antibodies to antigen and enhancement of immune complexation upon Congo red binding [45]. The curve shows increasing numbers of (radio-labeled) antiSRBC antibodies (derived from polyclonal serum) bound to the red cell as the concentration of Congo red increases. Congo red binding appears to enable low-affinity antibodies to form immune complexes

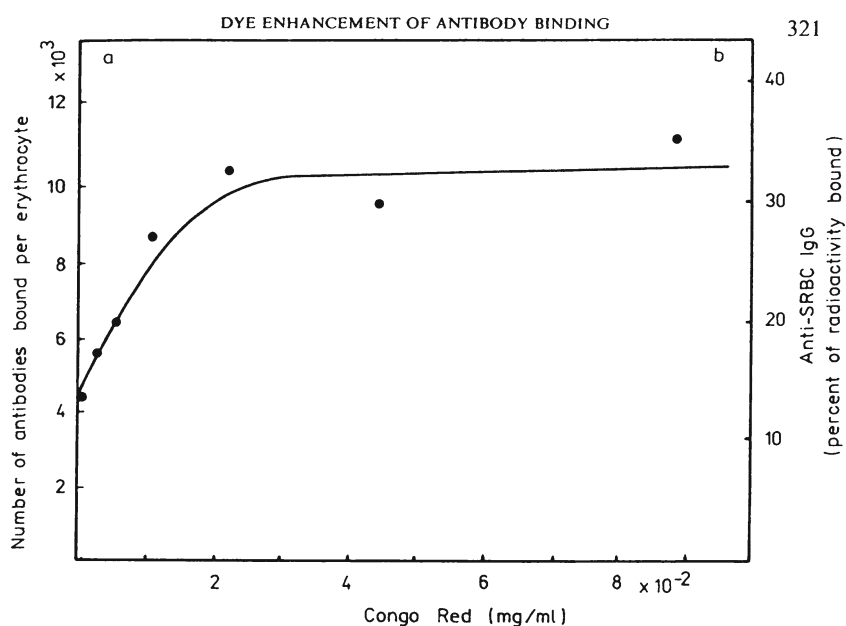
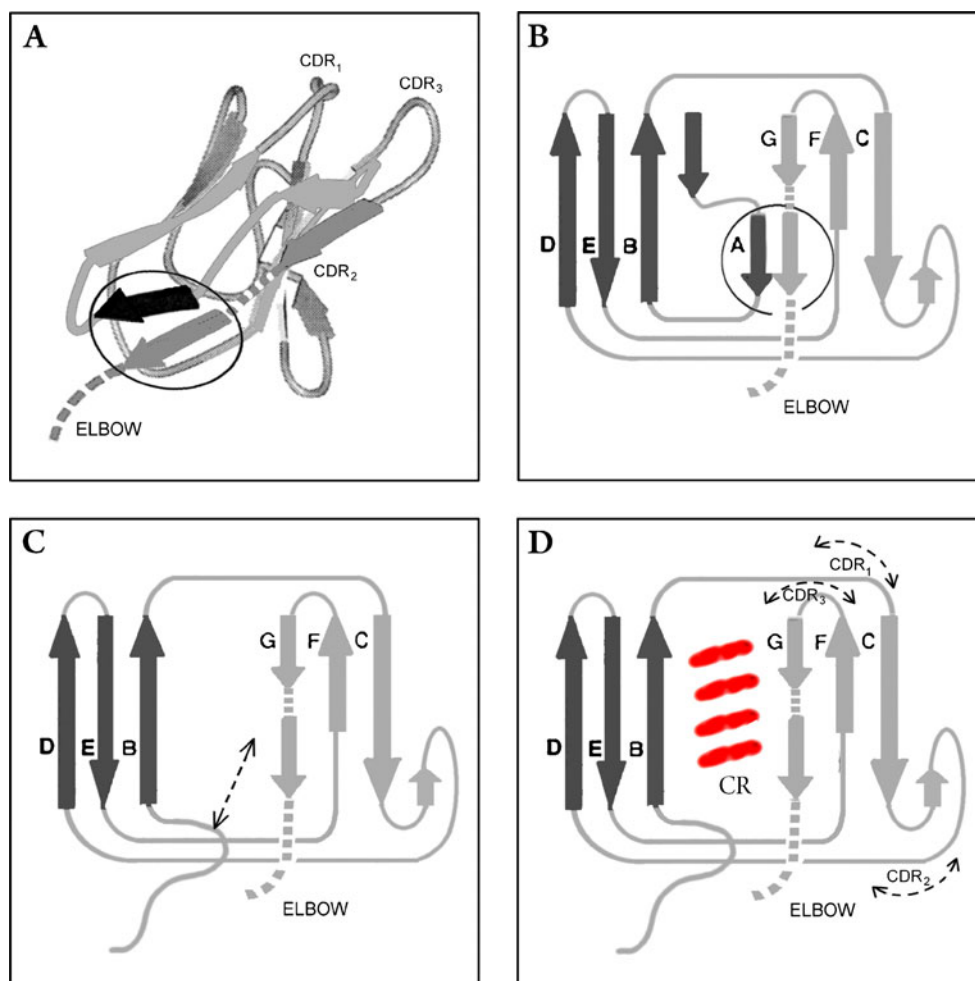


Fig. 6 Schematic view of the IgG L chain V domain showing the arrangement of polypeptide strands in β -sheets (a, b) and the alterations produced by the complexation of Congo red (c, d)



Convincing evidence that immune complexes — but not free antibodies — may become an acceptor for Congo red comes from observations concerning selective binding of dye by immune complexes in vivo. In vivo testing exposes

Congo red to highly diverse environmental conditions disturbing the carriage thus making the process reliable. As part of these studies, immune complexation was generated

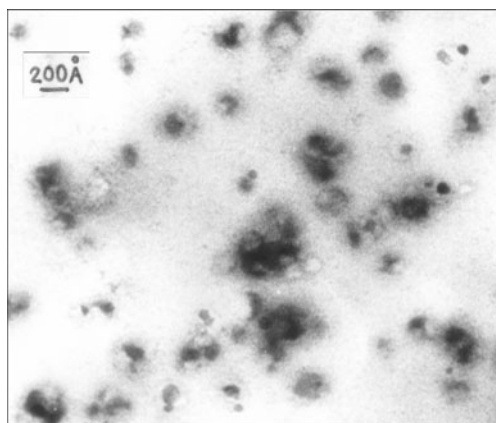


Fig. 7 EM picture of IgG (human) heated with Congo red in conditions standard for aggregation, isolated as high molecular weight fraction by SephatexG200 gel filtration. Single or aggregated molecules of IgG are seen as small irregular white spots within clouds of (silver contrasted) Congo red

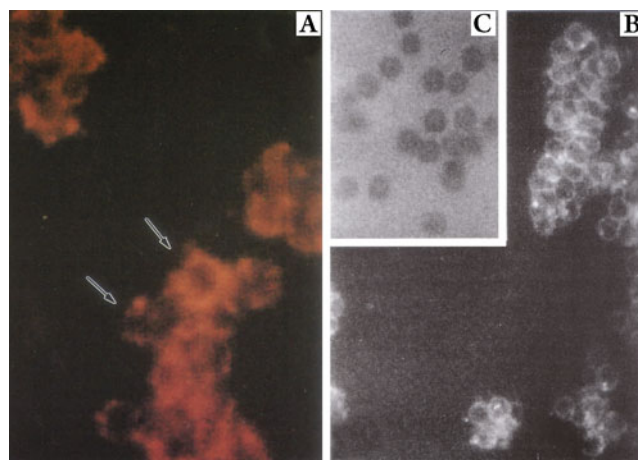


Fig. 8 The use of Congo red as a carrier transporting and introducing rhodamine B (bound by intercalation) to SRBC-antiSRBC immune complexes. Control: red cells treated with dye in the absence of antiSRBC antibodies (a sample in UV, b sample in UV presented in black and white, c control) [54]

locally in the ear of immunized rabbit by antigen injection (Arthus effect) [55]. This was followed by intravenous

injection of Congo red. Owing to the low opacity of rabbit ear tissues, the collection of dye in the locus of injection of

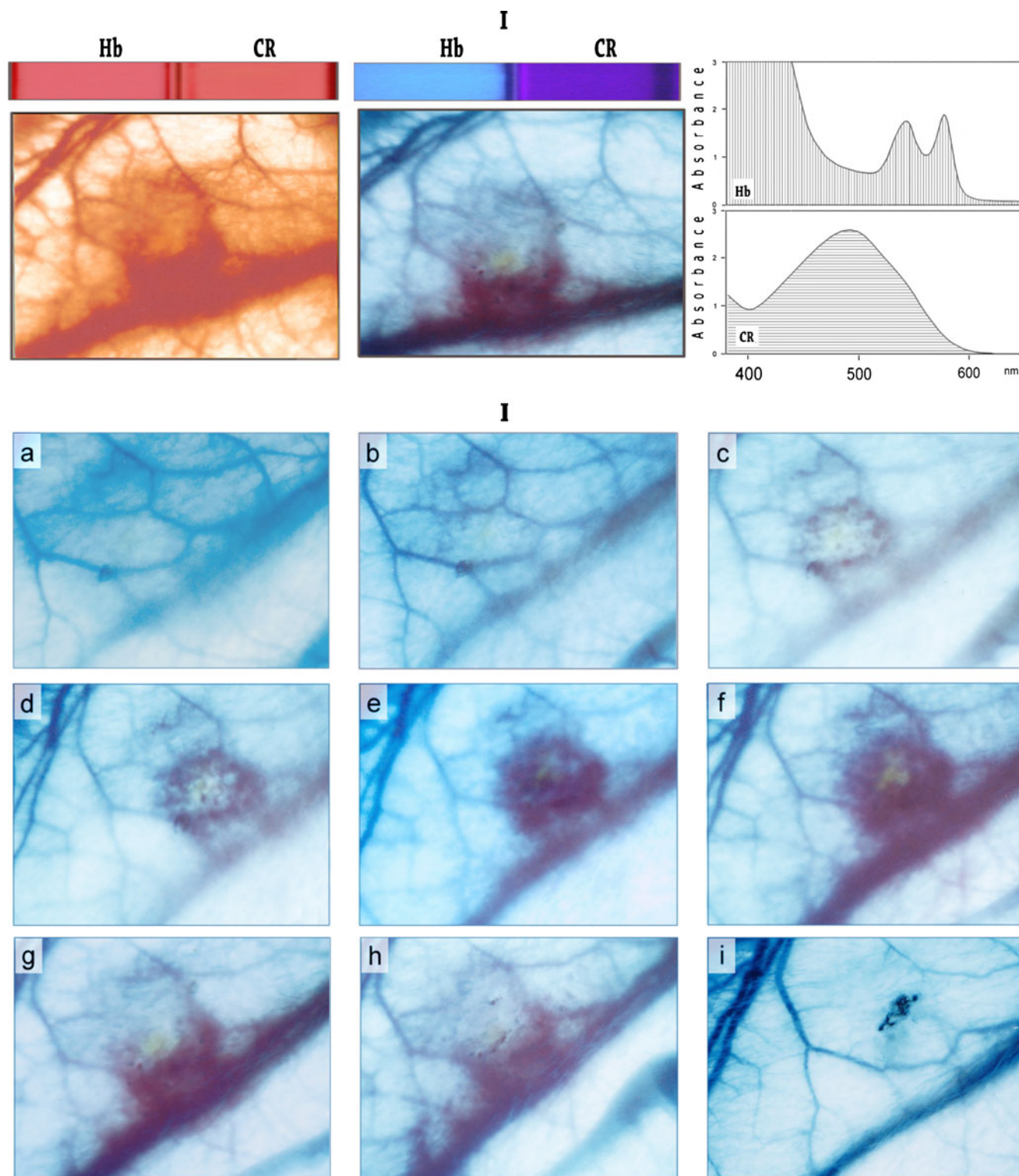
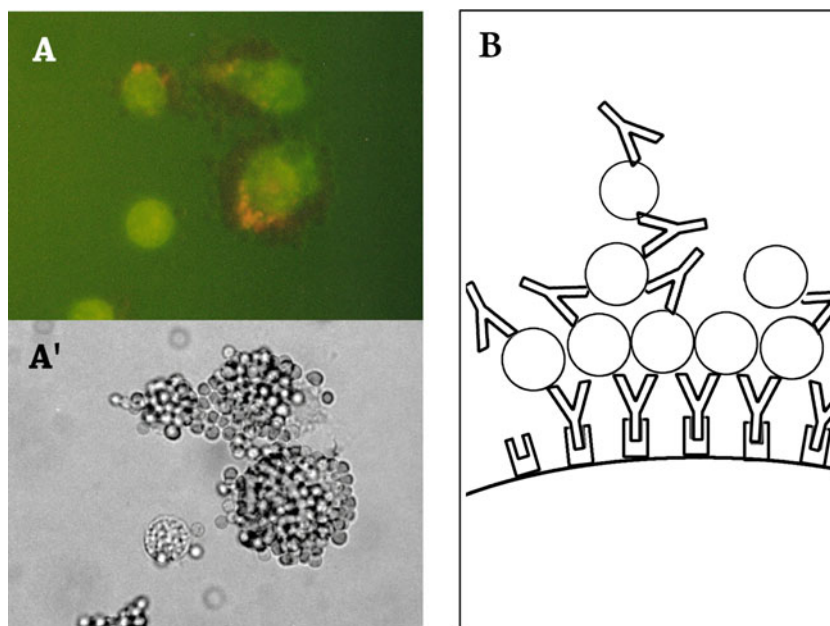


Fig. 9 The kinetics of Congo red binding to immune complexes upon local introduction of an antigen to the ear of immunized rabbit (Arthus effect) followed by intravenous administration of Congo red. A source of visible light was placed in the background and suitable color filters were selected to

differentiate hemoglobin and Congo red (top figure). **a** without filters **b** with filters **c** HbO₂ and Congo red spectra. **I** – The effect of using filters to differentiate HbO₂ and Congo red in solutions and directly in the ear of rabbit—top figure. **II** – Collection and dispersal of dye – bottom figure

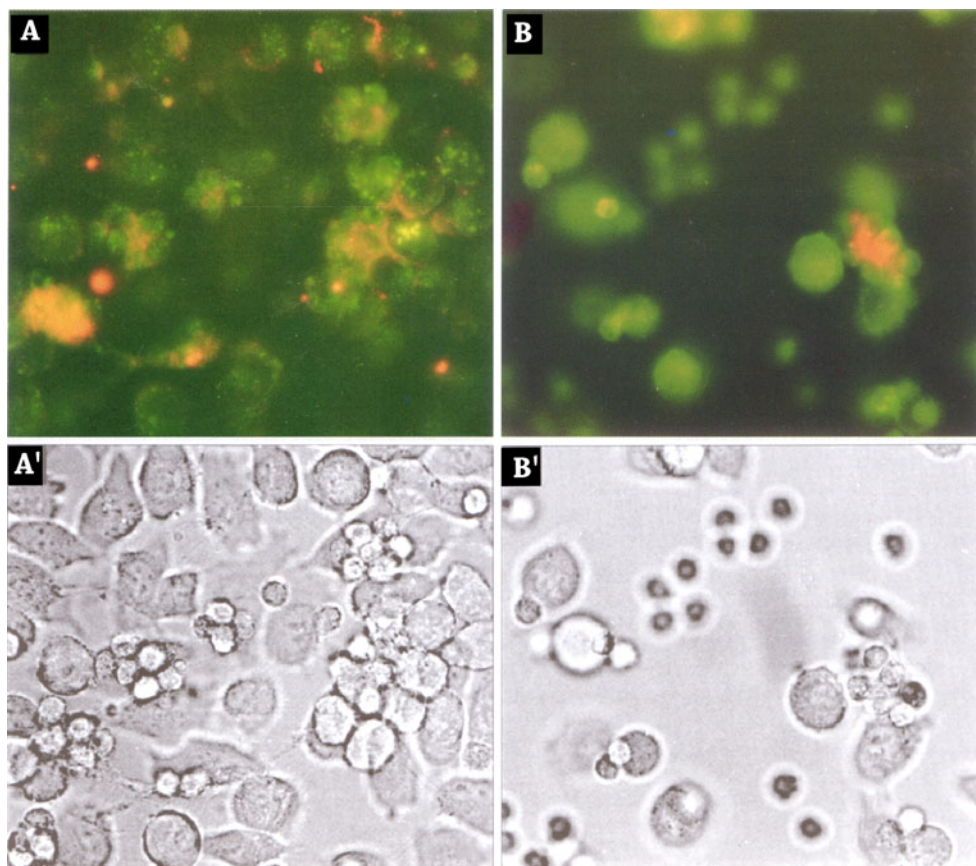
Fig. 10 Formation of rosettes (mouse monocytes 3774A1 in SRBC-antiSRBC system) via susceptibility of surface proteins to Congo red binding (**a** UV image; **A'** visible light image; **b** model view)



the antigen could be observed using a source of visible light. Pictures were taken using filters which enabled differentiation of HbO_2 and Congo red due to their differing spectra. As a result, shades of red (which cannot otherwise be differentiated without the use of filters) appear more blue for HbO_2 and more violet for Congo red (Fig. 9I) [56]. The

bottom part of Fig. 9II presents the collection and disappearance of Congo red from the vicinity of the antigen over time. The concentration of Congo red in the observed area increases over approximately 20–30 h and then slowly begins to decrease. The final picture was taken 3 weeks later.

Fig. 11 Binding of Congo red to surface proteins of interacting monocytes and cancer cells (HCV29T human and human monocytes). Fluorescence of Congo red registered in UV and visible light



Cell surface proteins interaction with Congo red

Congo red also attaches to special complexes formed by monocytes or T-lymphocytes and red cells (rosettes) in the presence of specific anti-red-cells antibodies [57–59]. Neither red cells nor monocytes bind the dye themselves. In the present experiment the dye is, however, attached to monocytes forming rosettes. Two kinds of immune complexes are formed in this experimental model. The former is based on antibodies affixed to Fc receptors distributed on the surface of monocytes, while the latter represents antibodies attached to red cells only. This confirms that structural changes in surface proteins engaged in complexation (antibodies – Fc receptors) are responsible for binding the dye. In the presented case, however, a portion of the attached Congo red was induced to fluorescent emission, which hinted at penetration of dye to more hydrophobic portions of interacting proteins [59]. This phenomenon only involves dye which is engaged in complexation directly on the surface of mouse monocytes (cell line J774A.1, ATTC TIB67) (Fig. 10) [59].

Microscopic pictures taken in UV and visible light reveals the nonhomogeneity of immune complexes formed as rosettes in the SRBC-antiSRBC system on the basis of monocytes as a source of Fc receptors. Congo red bound to primary antibodies affixed to Fc receptors appears to emit yellow and red fluorescence, while molecules attached to poorly ordered parts of immune complexes remain inactive. Such behavior seems to be connected with strain within proteins engaged in Congo red binding.

The induced fluorescence of Congo red was also observed to appear when the dye was added to human monocytes interacting with epithelial human bladder transformed cells (HCV29T, Polish Cellular and Molecular Biology Network, UNESCO/PAN) (Fig. 11) [60–64].

The dye binds and produces fluorescence only in conglomerates comprising both kinds of cells, suggesting their mutual interaction, induced strain and subsequent structural alterations of receptor proteins.

Conclusions

From among many different types of supramolecular structures, rode-like or ribbon-like molecular assemblies reveal an unusual capacity for penetration and binding to protein molecules. The nature of this interaction differs from that of typical ligands as the structures in question bind to areas which are not biologically predisposed to ligation. Moreover, their complexation produces functional enhancement rather than inhibition of the proteins' function. This mechanism can be explained on the basis of numerical analyses of the Congo red molecule structure (the dye chosen as the

model), supported by experimental studies [17, 27, 28, 30, 31, 35, 36, 44].

Having researched the problem for several years we believe we can now present some conclusions regarding this particular type of ligation. It appears to us that the unique properties of supramolecular assemblies of ribbon-like micellar structures forming nonstandard complexes with proteins and penetrating into their interior outside of the binding site are the result of:

1. Relatively large area of interaction (contact surface), ensuring sufficient binding strength for complexation;
2. Exposure of hydrophobicity along the ligand, favoring adhesion to polypeptide chains;
3. Plasticity of the supramolecular ligand which results from non-covalent stabilization and facilitating structural fitting.

To satisfy the binding conditions, the supramolecular ligand must exhibit:

- A – Sufficiently strong self-assembling tendencies to preserve the integrity of the ligand during complexation with the protein molecule;
- B – Sufficient spacing of charges in the ligand molecule, which is necessary to avoid conflicting interactions upon penetration into the protein interior.

The binding requirements related to the protein structure can be summarized as follows:

1. A necessary prerequisite for binding supramolecular ligands is a local structural instability, which may be caused by the presence of unfolding conditions or by structural alteration associated with the protein's function.
2. β -structures seem preferable as a binding target in proteins.

Understanding of the supramolecular ligand binding mechanism creates interesting application opportunities in biological studies and medicine.

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